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Specification Pages 25-47

Appendix A

EXAMPLES

Example 1.

The Effect of CO Exposure On Time Dependent Changes in Meat Color:

Samples of fresh meat (30 grams of beef, veal, or turkey) treated with CO by the method of the present invention were incubated in a suitable container for thirty minutes at a temperature of 15±3°C. Control samples were treated identically to CO-treated meats but were treated with air. The meat samples were removed from the container and were placed on an open benchtop at 15±3°C, or in an air exposed thermostatically controlled environment at 37°C. The color of the air treated meats turned brown gradually (within three hours at 37°C and twelve hours at 15±3°C), indicating non-fresh or spoiled meat. In contrast, the CO-treated meat samples maintained a wine-red color for at least 24 hours following exposure.

Example 2.

Comparison of CO, air, N_2 and vacuum treated meats

Fresh meat quarters (beef) were kept for six days at -2°C. The meat was then cut into 30 gram pieces (4" X 2" X 0.1") and divided into four groups and treated as follows: (A) vacuumed and (2-4) were introduced into gas tight containers by method previously described above and filled at a pressure of 1.1 atmospheres with gas at a volume which was ten time (10X) the volume of the meat. The gases used were: group D filled with CO, group B filled with N_2, and group C filled with air. A portion of the samples was kept at 15±3°C and the rest at 7°C. After twenty-four hours at 15±3°C and 48 hours at 7°C time dependent changes in color were observed. The samples of group A (maintained solely under vacuum) were brownish-purple. The samples of group B (N_2 treated samples) were brownish-red. The samples of group C (air treated samples) were brown. However, the color of the samples of Group D (CO treated) were unchanged remaining bright wine-red as shown in Figure 4.

Example 3.

Color Changes in Large Meat Chunks: Time Dependency During Meat Preservation

Beef chunks of 0.5 - 1.5 Kg were turned into CO-treated meat samples by treating with a 100% meat volume of gas according to the method of the present invention. Control chunks from the same source were treated identically but with air instead of CO. All of the chunks were kept at 4°C. The surface color of the air treated meat became brown after three days. The meat chunks were cut transversely for observation of color changes. Color change propagated with time in all meats from the surface towards the center of the chunk and were brown in air treated samples and wine-red in CO-treated samples. Following eighteen days of incubation, the air treated chunks were completely dark brown. In the CO-treated meat, the color change was 3-5mm from the surface after one and a half hours. Twelve hours post treatment a two cm ring of color change was observed. Three days post treatment, only five percent of the area of the transverse section remained unchanged. After seven days post treatment the color change was complete.

Propagation of the color from the surface into the interior of the chunks for chunks kept under 5% meat volume of gas was somewhat slower as compared to those chunks maintained kept under 100% meat volume gas. It is important to note that care should be taken to prevent adherence of any of the meat surface to the container.

Example 4.

Color Changes in Large Meat Chunks: Time Dependency After Removal From Container

An experiment similar to the previous example was performed except that the CO-treated meat chunks and the air treated meat chunks were removed from their containers after 21 days. The colors observed were the same as in the previous example. The chunks were left open to the atmosphere at 4°C. The color of the CO-treated was maintained for fourteen days. At day 14, only the surface (<1mm deep) of the CO-treated meat was brown. The color of the air treated meat remained dark brown throughout as shown in Figures 5 and 6.

Example 5.

Comparison of Bacterial Growth Under Air, CO and N_2 Atmospheres

Meat samples weighing 4.00±0.18 grams, were treated by flushing with either air, CO, or N_2. After twenty-four hours of storage at room temperature (15±3°C) each of the treated meat samples was soaked in sterile 0.15M sodium chloride (1ml per 2 grams of treated meat) for ninety minutes to extract any bacteria present on the surface of individual samples. Aliquots of serial dilutions of the extracts were plated onto non-selective agar plates (Bactoagar, Difco) and on Gram negative selective plates (MacConkey, Difco). All the plates were incubated over night at 37°C to allow bacterial growth. Bacterial colony counts per gram of meat (mean ± SD) are summarized in Table 1.

TABLE 1

Gas in Atmosphere	No. of colonies grown on bactoagar	gram negative
Air	$(1.42\pm0.3)\times10^6$	$(2.74\pm1.12)\times10^6$
N_2	$(1.63\pm0.3)\times10^6$	$(0.46\pm0.25)\times10^6$
CO	$(0.29\pm0.01)\times10^6$	$(0.02\pm0.01)\times10^6$

Example 6.

Toxicity and Edibility of CO-Treated Meat

Samples of CO treated meat of approximately 30 grams in weight or ground meat samples were stored for up to seven days at a temperature between 4 to 10°C. Samples of the CO treated meat were given to twelve starved cats (4kg per cat). Under these conditions, the meat samples were immediately consumed. No mal-effects were observed in any of the animals within 48 hours post consumption.

Additionally, four dogs each of which weighing approximately fifteen kilograms was offered 100 to 150 gram sample of CO preserved meat. The samples were consumed by the dogs and no mal-effects were observed in any dog within 48 hours after consumption.

Cats were fed fresh CO treated meat (250 grams per day per cat for one week). A control group was fed meat from the same source which was treated identically but with air instead of CO. The animals were continuously monitored by animal-tenders and showed regular behavior. The animals were found healthy by a house veterinarian. At the end of the experiment, a blood sample was drawn from all the animals and the red blood cells

were separated. The state of hemoglobin in these cells was analyzed spectrophotometrically as shown in Figure 3. From the spectra, it was found that the hemoglobin of both groups was completely in the oxy-hemoglobin form indicating no CO in the blood of the animals fed CO-treated meat.

Example 7.

Determination of Shelf-Life Extension by Bacterial Count as Limited by International Control Standards: Preservation at Room Temperatures.

General Methods:

Meat packing: Freshly slaughtered meat (beef) chunks of 0.5-1.5 Kg were cut into 25cm^2 pieces, 0.5-1.0 cm width (12-20 grams). Four samples were immediately submitted to bacterial count (as described below). The rest of the samples were treated with CO according to the method of the present invention. The gas pressure was 1.1 atmospheres and the volume of gas was equal to (100%) of the meat weight. The gas content was either air or 100% CO. The samples were preserved within a predetermined temperature range. Bacterial growth was measured at time intervals determined according to the temperature of preservation.

Evaluation Of Hemoglobin And Myoglobin Oxidation State In CO Treated Meats:

The quality of the CO-treated meats depends on the amount of globin fractions converted to CO bound forms and their location. The location is important since bacterial growth starts on the surface of the CO-treated meats as does CO penetration. Assessment of the CO bound myoglobin and hemoglobin in the CO-treated meats was carried out using two parameters: (a) measurements of the circumference width of zones which underwent visible color change due to CO binding (CO-treated meats were successively cut transversely and the depth of the color-changed zone was measured with a ruler) and (b) assessment of the CO bound fraction of hemoglobin and myoglobin in CO-treated samples.

Procedure: Samples of the CO-treated meats (1 to 5 grams) were homogenized in an equal volume of phosphate buffer (0.1M, pH 7.4) to extract both hemoglobin and myoglobin. The extract was centrifuged at 40,000 g for ten minutes. The supernatant was isolated and its absorption spectrum was measured in the 400 to 700nm range. From the position of the absorption peaks and their relative heights the fraction of CO bound globins was calculated.

Bacterial growth measurement was made according to international standards and carried out by a ISO 9000/IEC Guide 25 licensed bacteriological laboratory. As bacterial growth is mostly on the meat surface, the routine contamination tests at governmental laboratories relate to bacterial growth on a standard minimal area of $25cm^2$. The bacterial growth is then expressed as the number of bacteria per cm^2. The most stringent standards allow a growth of up to $5X10^6$ (6.7 in log scale) bacteria per cm^2 while the least strict ones consider a growth of up to $1X10^7$ (7.0 in log scale) non-contaminated.

The procedure entails treating a 25 cm^2 surface area sample of the meat with 25 ml of aqueous solution. The bacterial content is introduced into the solution using a stomacher apparatus (Seward Lab U.K.). This suspension is then diluted in a ten fold series up to 10^{-9} in 0.1 M phosphate buffer, pH 7.0. One ml of each dilution is applied to each of three types of 60mm growing plates: (a) containing plate count agar (PCA, Difco) incubated at 33°C±0.2 for 48 hours to enable total viable aerobic count, (b) containing SPS agar (Difco) allowing clostridium growth, and (c) containing the same medium as a (a) allowing

microaerophile growth. Type (b) and (c) plates were confined within sealed anaerobic jars supplied with gas generating kits (Oxoid, U.K.) and incubated for twenty four hours at 35°C. Bacterial colonies (up to 200 per plate) were counted using a colony counter.

According to international health standards, the maximal bacterial growth allowed for non-contaminated meat is $1X10^7/cm^2$ total viable aerobic bacteria and $1X10^4/cm^2$ of microaerophils. The shelf life of a meat in a non-contaminated form was determined by the duration until the above defined bacteria levels were reached (<u>"preservation duration"</u>).

Twenty experiments were carried out as follows: meats samples were preserved at 5 ± 3.5°C and bacterial counts were carried out at intervals of four days. The length of "preservation duration" in these experiments (expressed as mean $\pm$SE) were for microaerophils: 8.72 ± 2.1 days for air treated samples and 18.9 ± 3.27 for CO-treated samples (see Figure 2a); by the criteria of total viable aerobic bacteria 11.13 ± 1.11 days for air treated meat samples and 23.12 ± 2.79 for CO-treated meat samples (see Figure 2b).

This data shows that the meat preservation method of the present invention succeeded in extending the shelf life more than two fold.

Example 8.

Determination of Meat Shelf-life Extension by Bacterial Count as Limited by International Control Standards: Preservation at High Room Temperature

As in the previous example, meat samples were preserved at 26±4°C. Due to the high temperature which accelerates bacterial growth, bacterial count was determined at five hour intervals. Four experiments were carried out and the length of "preservation duration" in these experiments (expressed as mean ±SE) were: by the criteria of total viable aerobic bacteria 13±1 hours for air treated samples and 30±1 hours for CO-treated samples (see Figure 1). By the microaerophils count criteria: 9.5±0.9 hours for air treated samples and 9.5±0.8 hours for CO-treated samples.

Example 9.

Inhibition Of Bad Odor Development In CO Treated Meats

In all meats preserved as in examples 1 through 8, it was found that by the time of detection of bad odors in air preserved meats, no similar odor was detected in the CO-treated meats. This finding indicates that the arrest of meat-spoilage was achieved by the meat preservation method of the present invention. Consistently, in all preserved meat samples bad odor was detectable in samples which showed high count of bacteria. Additionally, by the time bad odor was detectable by the average human nose, the total viable aerobic count exceeded 10^8 colonies/cm^2, a value which exceeds that allowed for non-contaminated meats (10^7 colonies/cm^2). Thus, odor is a less sensitive indicator of meat spoilage than bacterial count. However, odor is valuable because it can be used by most people including the meat consumers.

Table 2 represents the "preservation duration" under various conditions at which CO-treated meat retained pleasing odors while air preserved meat smelled badly.

TABLE 2

Type of meat	Size	Temperature range of preservation, °C	"Preservation duration in days
Beef	Slices	2-9	18
		22-30	3
Beef	Chunks	2-9	18
	Chunks	14-17	7
Veal	Slices	22-30	3
Turkey	Slices	22-30	3

Example 10.

CO Diffusion From Meat Surface into the Core During the Preservation

Meat samples were sealed in plastic bags under 100% CO at a pressure of about 1.1 atmosphere. CO volume was 30 ± 20% of the meat volume. The bags were kept at 5°C. Figures 7-9 demonstrate a typical experiment in which meat was treated 24 hours after slaughter. A sample of calf meat weighing 18 pounds (about 8 Kg) was cut into two nine pound pieces having maximum length of 30 cm and maximum width of 20 cm. One of these samples

was kept frozen at -18°C while the other was preserved in CO at 5°C. After three days, the bag was opened to release the unbound CO and the meat was left in the open air for half an hour. The meat sample was cut transversally at about one third of its length (7 cm from one edge). About 30% of the meat radius (from surface to core) changed in color from dark to bright red (see Figure 7). Because any CO which reaches Hb/Mb bind quickly, the change in meat color serves as a measure of the CO diffusion rate.

The meat samples were reassembled and repacked with CO. Following seven additional days at 5°C, the meat samples was again unpacked and cut transversally once in the middle of the sample, about 15cm from the edge (shown in Figure 8), and once closer to the end of the sample, at about one third of the sample's length (shown in Figure 9). As shown in the transverse cut in Figure 8, the meat was almost completely bright red except for a small dark red area shown by an arrow. This indicates a nearly complete diffusion or saturation of the CO from the surface to the core of the meat sample. The cut at one third of the sample's length shows that in this zone, oxy-Hb/Mb were completely converted to their carbomonoxy forms as

shown in Figure 9. The meat samples were reassembled and kept at 5°C under CO for an additional two days to ensure complete conversion of Hb/Mb to the carbomonoxy forms. After 12 days under CO at 5°C, the color of a middle sample (transverse cut) differed dramatically from that of the frozen sample (see Figure 10), indicating that oxy-forms of Hb/Mb were preserved in the frozen meat sample (Figure 9, right) while carbomonoxy forms prevailed in the CO treated meat (Figure 10, left).

Example 11.

Appearance of the Cooked Meats

From the 12 day-old, frozen and CO treated meats (shown in Figure 10), 5cm in width pieces were cut transversally. Each piece was cooked in 100 ml distilled water in a covered pot on a low flame for 120 minutes. At the end of the cooking period, all of the water had evaporated. The appearance of the cooked meat pieces is shown in Figure 11. They were similar except for some browning of the surface of the CO treated meat due to complete water loss in the meat.

To reveal the meat interior, the two pieces were cut transversally. A small test by ten different people assured that pieces smelled alike having a typical cooked meat smell. The transverse sections are seen in Figure 12 and were very similar in appearance. This experiment demonstrates the loss of the bound CO during the cooking process (heating) which leads to protein denaturation and heme degradation.

Example 12.

Measurements of CO Release in the Process of Cooking

A. Experimental procedure:

All measurements of CO release in the process of cooking were carried out in a 7.5 liter sealed regular pressure pot ("pressure cooker") to which manometer/thermometer was inserted to measure the temperature and pressure during cooking. The pot was also equipped with a controlled outlet (valve). To ensure complete sealing the pot lid was also smeared with a layer of high vacuum grease prior to locking the pot. The pot was then heated by cooking gas for a few minutes until reaching a pressure of 1.5 atmospheres and a temperature of 235°F (113°C). At this stage the pot was transferred to an electric heater for further

cooking keeping the pressure within the pot at 1.7±0.7 atmospheres and the temperature at 114± 12°C. Cooking time varied among experiments within the time range of regular domestic cooking, namely 60-140 minutes. The pot was allowed to cool until reaching room temperature and then was connected via the valve to a CO monitor. The CO level in the pot atmosphere was expressed in PPM.

The measure CO level in PPB(m) was translated and expressed as CO PPM(c) released from 2 Kg of cooked meat (a family meal size) into a sealed room of 3.0 square meters (kitchen size dimensions). Considering the volume of one mole of gas at room temperature as 22.4 liters, the expected CO level was calculated as:

$$PPM(c) = 0.074 . PPM(m)/X$$

Where: PPM(c) = calculated PPM;
PPM(m) = measured PPM;
X = meat weight in grams.

B. Safety in cooking the CO treated meat:

200-650 gram meat samples (either in one piece or ground) were treated with CO and stored within a confined CO atmosphere for 14 days at 5°C. The meats were cooked within 30 minutes of removal from the CO packaging bags. The results (expressed

as explained above) are summarized in Table 3. As can be seen, the average PPM(c) level given as Mean ± S.D. (N) was 0.056 ± 0.026 (10). In all experiments, less than 0.1 PPM of CO was released from 2 Kg cooked meat into the hermetically closed room. Since the TLV for CO is 25 PPM, it can be seen that the released CO level from the cooked meat (2Kg) in our experiments, was far below that safety limit. In fact it is low enough not to alarm a domestic CO detector (100 PPM for 90 minutes in a regular room size). Thus, under our experimental conditions, the consumer is not exposed to danger. Moreover, treated meats could be removed from their CO packing bags and maintained for at least 14 days in the open air at 5°C without any risk of CO release prior to cooking.

No statistically significant test analysis could be made because the experimental conditions were incomparable (chosen to cover various cooking conditions). However, there is a trend showing that the level of released CO is inversely correlated with the post-slaughter period until CO treatment began. Thus, the sooner (closer to slaughtering) the meat was treated, the higher the CO level (see the declining order in items 1-4

in ground meat in Table 3). Note that CO release level reflects the CO bound level in the meat. These findings agree with a slow autooxidation of Hb/Mb in meats exposed to air prior to CO packing, resulting in formation of met-Hb/Mb which can no longer bind CO. Therefore, upon packing, less CO will be bound and consequently less CO will be released later upon cooking. The data from the experiments (items 6-8a in Table 3) suggest that up to a period of two days post-slaughter, CO release level was similar.

From items 8a-8c (Table 1), wherein the portions of the same CO treated sample were removed from the CO packing bag and exposed to open air for various periods, it appears that the CO was maintained within the samples for at least three days.

Example 13.

Release of CO by Cooking

Freshly drawn human blood was used. The red cells were washed and lysed in a hypotonic buffer. The mixture was centrifuged to separate cytosol from membranes. The concentration of hemoglobin in the solution was measured spectrophotometrically and was found to be 16.2 mM.

The solution was sealed in a beaker and CO was gently flushed above the solution surface while stirring for 30 minutes. A few grains of dithionite was added to consume any residual oxygen. The color of the solution turned bright red typical of carbomonoxy Hb. The solution was then left while stirring (for 15 minutes) to the open air to released dissolved CO. The Hb was identified spectrophotometrically as carbomonoxy Hb. The hemoglobin solution was cooked exactly as the meat and the amount of CO in the pot atmosphere was measured. From the concentration of hemoglobin (each heme molecule binds one CO molecule) the amount of CO molecules was calculated in the solution. From this amount and the pot volume, the expected PPM level of totally dissociated CO was calculated. As in the procedure of cooked meats the gas level in the pot was measured by the CO monitor. The ratio of measured to calculated CO level turned out to be 1.06 indicating that, within experimental error, all CO was indeed released from the Hb by cooking.

TABLE 3: CO RELEASE FROM COOKED MEATS

Item No	Meat form	Post Slaughter period to treatment (days	PPM(c) of CO
1)	Ground	6	0.052
2)	Ground	8	0.039
3)	Ground	8	0.039
4)	Ground	10	0.013
5)	Ground	1(14)*	0.033
6)	Chunk	0	0.093
7)	Chunk	0	0.053
8a)	Chunk	2	0.071
8b)	Chunk	2(1)*	0.076
8c)	Chunk	2(2)*	0.088

All meats (200-650 gr) were treated with CO and were stored in CO atmosphere for 14 days at 5°C. The meats were cooked within 30 minutes after exposure to air. (*) refers to exposure period at 5°C (in days) in the open air following removal from CO packaging bags. Items 8a-c are sub-chunks which were left in the open air for different time periods. Average PPM(c) = 0.056 ± 0.026

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

REFERENCES CITED

Baranaga, "Carbon Monoxide: Killer to Brain Messenger in one Step", Science, 259, 309 (1993).

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Claim Pages 48-53

Appendix B

CLAIMS

What is claimed is:

1. A method for preserving meat by: exposing raw meat to an atmosphere consisting essentially of carbon monoxide and maintaining the meat in a vacuum free, sealed container to maintain color and freshness while retarding bacterial growth.

2. A method as set forth in claim 1 wherein said exposing step is further defined as completely immersing the meat throughout with an atmosphere consisting essentially of carbon monoxide.

3. A method as set forth in claim 1 wherein said exposing step is further defined as exposing the meat to carbon monoxide gas for approximately 1 to 30 minutes prior to sealing the container.

4. A method as set forth in claim 3 wherein said exposing step is further defined as exposing the meat to carbon monoxide gas for approximately five minutes prior to sealing the container.

5. A method as set forth in claim 1 wherein said exposing step is further defined by exposing the meat to a volume of carbon monoxide ranging from approximately 5 to 100 percent by weight or volume of meat being treated.

6. A method as set forth in claim 5 wherein said exposing step is further defined by exposing the meat to a volume of carbon monoxide of approximately thirty percent by weight or volume of meat being treated.

7. A method as set forth in claim 1 wherein said exposing step is further defined as exposing the meat to carbon monoxide gas between a temperature range of approximately -2 to 37°C.

8. A method as set forth in claim 1 wherein the sealed container is a plastic bag.

9. A method as set forth in claim 1 wherein the sealed container is a sealed chamber.

10. A method as set forth in claim 1 wherein the carbon monoxide in the container has an atmospheric pressure of approximately 1.5 to 5 atmospheres.

11. A method as set forth in claim 10 wherein the carbon monoxide in the container has an atmospheric pressure of approximately 2.0 atmospheres.

12. A method as set forth in claim 1 further including the step of storing the meat at a temperature above freezing following said carbon monoxide exposure step.

13. A method as set forth in claim 12 wherein the temperature is further defined as a temperature range from approximately -2 to 30 °C.

14. Meat treated and preserved by the process comprising the steps of:

exposing raw meat to an atmosphere consisting essentially of carbon monoxide and maintaining the meat in a vacuum free sealed container to maintain color and freshness while retarding bacterial growth.

15. Meat treated and preserved by the process according to claim 14 wherein said exposing step is further defined as completely immersing the meat throughout with an atmosphere consisting essentially of carbon monoxide.

16. Meat treated and preserved by the process according to claim 14 wherein the exposing step is further defined as exposing the meat to carbon monoxide gas for approximately one to thirty minutes prior to sealing the container.

17. Meat treated and preserved by the process according to claim 14 wherein the exposing step is further defined by exposing the meat to a volume of carbon monoxide for approximately five minutes prior to sealing the container.

18. Meat treated and preserved by the process according to claim 14 wherein the exposing step is further defined as exposing meat to a volume of carbon monoxide ranging from approximately 5 to 100 percent by weight or volume of meat being treated.

19. Meat treated and preserved by the process according to claim 14 wherein the exposing step is further defined as exposing meat to a volume of carbon monoxide of approximately 30 percent by weight or volume of meat being treated.

20. Meat treated and preserved by the process according to claim 14 wherein the sealed container is a plastic bag.

21. Meat treated and preserved by the process according to claim 14 wherein the sealed container is a sealed chamber.

22. Meat treated and preserved by the process according to claim 14 wherein the carbon monoxide in the container has an atmospheric pressure of approximately 1.5 to 5 atmospheres.

23. Meat treated and preserved by the process according to claim 21 wherein the carbon monoxide in the container has an atmospheric pressure of approximately 2.0 atmospheres.

24. Meat treated and preserved by the process according to claim 14 further including the step of storing the meat at a temperature above freezing following the carbon monoxide exposure step.

25. Meat treated and preserved by the process according to claim 14 wherein the temperature is further defined as a temperature range from approximately -2 to 30 °C.

26. Meat treated and preserved by the process according to claim 14 wherein said exposing step is further defined as exposing the meat to carbon monoxide gas between a temperature range of approximately -2 to 37°C.